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Pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amides as novel antiproliferative agents: Exploration of core and headpiece structure–activity relationships

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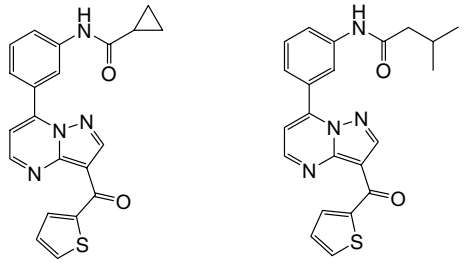
Abstract—A novel series of antiproliferative agents containing pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amides, selective for p21-deficient cells, were identified by high-throughput screening. Exploration of the SAR relationships in the headpiece, core, and tailpiece is described. Strict steric, positional, and electronic requirements were observed, with a clear preference for both core nitrogens, a thienoyl headpiece, and meta substituted tailpiece.

Progression from one phase of the cell division cycle to the next phase is controlled by a series of sensors and arresting mechanisms called checkpoints.[1] Loss of checkpoint control is a hallmark of tumor cells, as it increases the mutation rate and allows a more rapid progression to the tumorigenic state.[1,2] However, inactivation of these checkpoints results in aberrant responses to cellular damage. This failure of checkpoint responses in malignant cells can be exploited in cancer drug discovery. Identification of compounds that selectively kill checkpoint-deficient cells compared with checkpoint-proficient cells can be expected to preferentially target tumor cells, while sparing normal cells.[3,4]

The p53 tumor suppressor gene is the major regulator of the DNA damage checkpoint and one of the most commonly mutated genes in human cancer (50–70%).[5,6] As a downstream effector of p53, p21 inhibits the cyclin-dependent kinases (CDKs) and arrests cell cycle progression in response to DNA damage.[7,8] Disruption of this checkpoint by deletion of the p21 gene in the HCT116 colorectal carcinoma cell line leads to the failure of the cell to arrest in response to DNA damage, endoreduplication, and ultimately, apoptosis.[9] These p21-deficient cells show increased chemosensitivity,[10] compared with the isogenic p21-proficient parental cells, to a variety of DNA damaging agents, including clinically used antineoplastic drugs. We have used this isogenic pair of cell lines [HCT116 (p21+/+) and 80S14 (p21−/−)[12]] to identify novel molecules that preferentially induce apoptosis in the p21-deficient cells.

High-throughput screening[11] led to the identification of pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amide **1** (Fig. 1) which had an IC_{50} of 0.45 μM in the p21-deficient cell line and an IC_{50} of 11 μM in the p21-proficient cell line



1 **2**

Figure 1. High-throughput screening lead, **1**, for identification of p21 chemoselective agent and optimized amide analog, **2**.

Keywords: P21 deficient; Antiproliferative; Structure–activity relationship; Checkpoint.
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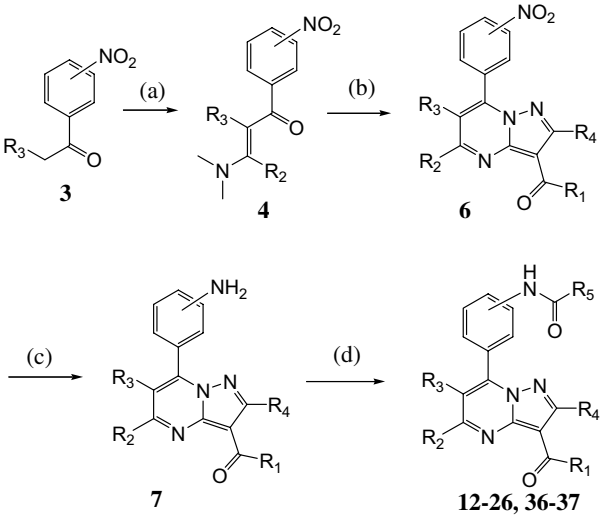
giving a selectivity ratio[11] of 23. Subsequent efforts explored the structure–activity requirements for the amide region of the molecule.[13] These efforts led to the identification of the isobutyl amide **2** with improved potency as well as selectivity (Table 1). The corresponding isopropyl carbamate and urea of **2** (not shown) also showed improved properties compared to **1**.[13] Our efforts to probe the steric requirements of the core and aryl ketone headpiece, attachment position for the tailpiece as well as the requirement for both core nitrogens are described below.

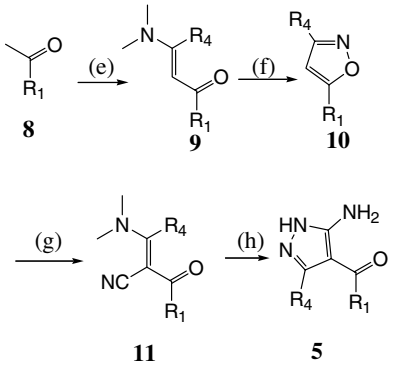
The majority of the compounds for this series were readily prepared[13,14] utilizing the syntheses shown in Schemes 1 and 2. In Scheme 1 *ortho*, *meta* or *para*-nitro substituted aryl ketones **3** were converted into the corresponding enamines **4** with either *N*,*N*-dimethyl formamide dimethyl acetal [DMFDMA] ($R_2 = H$) or *N*, *N*-dimethylacetamide dimethyl acetal [DMADMA] ($R_2 = CH_3$). Condensation of **4** with the amino pyrrazole **5** (synthesis shown in Scheme 2) afforded the cyclized nitro compound **6**. The reduction of **6** afforded the amine **7**. The reaction of **7** with cyclopropyl carbonyl chloride, isobutyl carbonyl chloride, isopropyl chloroformate or isopropyl isocyanate gave compounds **12**–**26**, **36** and **37**.[15]

Scheme 2 outlines the synthesis of the amino pyrrazoles **5**. Reaction of aryl ketones **8** with either DMFDMA ($R_4 = H$) or DMADMA ($R_4 = CH_3$) afforded the corresponding enamines **9**. Condensation of **9** with hydroxylamine followed by ring opening with DMFDMA afforded the enamines **11** which were condensed with hydrazine to afford the pyrrazoles **5**.

In order to explore whether both pyrimidine nitrogens were necessary to provide good activity, the pyrazolo pyridines, **32**–**35**, were synthesized as shown in Scheme 3. Amination of pyridine **27** afforded the amine salt **28**. Condensation of **28** with the alkyne **29** gave the cyclized compound **30**. Reduction of **30** afforded the intermediate amine **31**, which was acylated in the same manner as **7** to afford compounds **32**–**35**.



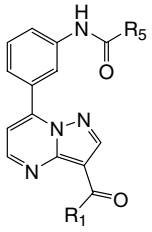
Scheme 1. Reagents and conditions: (a) DMFDMA or DMADMA, reflux, 18 h; (b) **5**, acetic acid, reflux; (c) Fe, NH_4Cl, MeOH, H_2O; (d) for **12**–**17**, **21** R_5COCl, pyridine, 0 °C to rt; for **18**, **22**, **25**, **36**, **37** R_5OCOCl, pyridine, 0 °C to rt; for **20**, **24** R_5NCO, pyridine, 0 °C to rt.



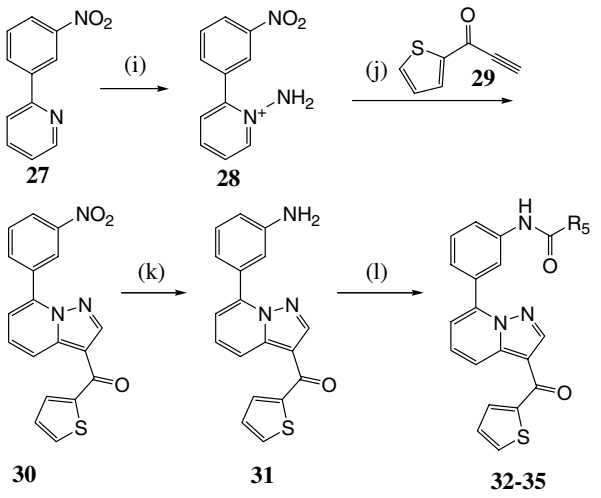
Scheme 2. Reagents and conditions: (e) DMFDMA or DMADMA, reflux, 18 h; (f) NH_2OH–HCl, reflux, 3 h; (g) DMFDMA, reflux, 18 h; (h) NH_2NH_2–H_2O.

Our initial efforts were focused on understanding the steric and electronic requirements of the aryl ketone,

Table 1. Activity comparison of aryl ketone derivatives in p21-proficient (p21+/+) and -deficient (p21−/−) cell lines



Compound	R_1	R_5	p21+/+ (IC_{50} μM)	p21−/− (IC_{50} μM)	Ratio
1	2-Thienyl	Cyclopropyl	11	0.45	23
2	2-Thienyl	*iso*-Butyl	6.4	0.14	46
12	Phenyl	Cyclopropyl	>20	8.1	>2.5
13	2-Furyl	Cyclopropyl	>20	3.6	>5.6
14	5-Methyl-2-thienyl	Cyclopropyl	>20	7.2	>2.8
15	2-Thiazole	Cyclopropyl	16.9	1.1	15
16	2-Pyridyl	Cyclopropyl	>20	>20	—



Scheme 3. Reagents and conditions: (i) *O*-mesitylsulfonylhydroxylamine, CH_2Cl_2, rt, 20 h, 67%; (j) K_2CO_3, DMF, rt, 12 h, 41%; (k) Fe, NH_4Cl, MeOH, H_2O, 60%; (l) for **32**, **34** R_5COCl, pyridine, 0 °C to rt; for **33** R_5OCOCl, pyridine, 0 °C to rt; for **35** R_5NCO, pyridine, 0 °C to rt.

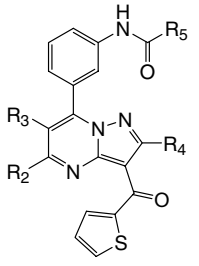
R_1 (Table 1). Conservative replacement of the 2-thienyl group with phenyl (**12**) resulted in a large decrease in cell potency. Heterocyclic replacements for 2-thienyl including five-membered ring isosteres 2-furyl (**13**), 2-thiazole (**15**) as well as 2-pyridyl (**16**) also led to a reduction in potency in both cell lines with 2-thiazole retaining the most potency and selectivity for p21−/− cells. Introduction of a 5-methyl onto the thiophene (**14**) led to a reduction of potency and selectivity. Replacement of the entire aryl ketone with a nitrile or ethyl ester (data not shown) led to a complete loss of activity in both cell lines. These initial studies indicated a strict electronic and steric requirement for good activity in this portion of the molecule.

We then focused our attention on exploring the steric requirements in the pyrazolo[1,5-*a*]pyrimidine core by introducing a methyl probe at all of the open positions (Table 2). For comparison purposes we prepared each methyl analog with the corresponding most potent R_5 substituents described earlier.[13] Introduction of a methyl group adjacent to the biaryl linkage, $R_3 = CH_3$ (**21**–**24**), led to an almost complete loss in potency in both cell lines. In addition, introduction of a methyl group adjacent to the aryl ketone, $R_4 = CH_3$ (**25** and **26**), also led to a decrease in potency. Both of these substitutions probed the steric requirements in their respective portions of the core, also causing an increase in the dihedral angles of the adjacent flanking substituents to avoid a steric clash with the methyl group. Either of these factors could be responsible for the change in potency. Introduction of a methyl group next to the pyrimidine nitrogen, $R_2 = CH_3$ (**17**–**20**), had relatively little effect on potency and selectivity with the biggest loss seen for the *iso*-butyl analog, **19**, when compared to its corresponding analog **2**. This would indicate that not only is there more space to introduce substituents in this portion of the core, but that access to the pyrimide nitrogen may not be crucial to activity.

With this information in hand we explored the requirement of the non-bridgehead pyrimidine nitrogen for activity (Table 3). The de-aza compounds (**32**–**35**) showed a >10-fold loss in potency in the p21−/− line and a corresponding loss in selectivity. Although the methyl probe results suggested only a minor role for a direct binding interaction of the pyrimidine nitrogen in activity, there appears to be a significant electronic effect of this pyrimidine nitrogen on the rest of the core that influences activity.

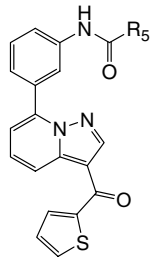
We also explored the positional requirements of the attached amide by utilizing the potent isobutyl amide

Table 2. Activity comparison for methyl substituted derivatives in p21-proficient and -deficient cell lines



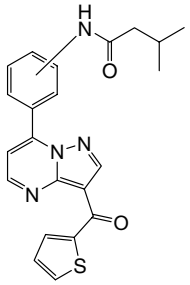
Compound	R_2	R_3	R_4	R_5	p21+/+ (IC_{50} μM)	p21−/− (IC_{50} μM)	Ratio
17	CH_3	H	H	Cyclopropyl	9.2	0.64	14.4
18	CH_3	H	H	*O*-*iso*-Propyl	3.9	0.28	14
19	CH_3	H	H	*iso*-Butyl	3.7	0.48	7.7
20	CH_3	H	H	NH-*iso*-propyl	11	0.62	18
21	H	CH_3	H	Cyclopropyl	>20	>20	—
22	H	CH_3	H	*O*-*iso*-Propyl	>20	17.3	>1
23	H	CH_3	H	*iso*-Butyl	>20	>20	—
24	H	CH_3	H	NH-*iso*-propyl	>20	>20	—
25	H	H	CH_3	*O*-*iso*-Propyl	>20	>20	—
26	H	H	CH_3	*iso*-Butyl	>20	>20	—

Table 3. Activity comparison for de-aza derivatives in p21-proficient and -deficient cell lines



Compound	R_5	p21+/+ (IC_{50} μM)	p21−/− (IC_{50} μM)	Ratio
32	Cyclopropyl	12.8	6.4	2
33	*O-iso*-Propyl	>20	2.4	>8.3
34	*iso*-Butyl	>20	4.2	>4.8
35	NH-*iso*-propyl	>20	5.6	>3.8

Table 4. Activity comparison for phenyl positional isomers in p21-proficient and -deficient cell lines



Compound	Position	p21+/+ (IC_{50} μM)	p21−/− (IC_{50} μM)	Ratio
36	Para	>50	>50	—
37	Ortho	>50	>50	—
2	Meta	6.4	0.14	46

Table 5. Activity comparison in Colon carcinoma cell line panel

Compound	LoVo (IC_{50} μM)	SW620 (IC_{50} μM)	DLD1 (IC_{50} μM)	HT29 (IC_{50} μM)
1	0.068	0.11	0.085	0.078
2	0.021	0.045	0.042	0.036
19	0.076	0.11	0.084	0.078
33	0.70	0.65	0.85	0.62

group (Table 4). Movement of the amide to the para and ortho positions led to loss in activity.

The most potent compounds from the p21−/− cell line were tested in a panel of colon carcinoma cells (Table 5). The overall potency trends seen in the p21−/− cell line were also seen in the colon panel, with the de-aza analog, **33**, being the least potent. The underlying mechanism for the preferential killing of p21−/− cells is not currently understood. However representative compounds in this series have shown an ability to bind to the colchicine binding site of tubulin.[16]

In conclusion, we have explored some of the steric, electronic, and positional requirements of the lead series. For the aryl ketone region of the molecule a strict requirement for an unsubstituted 2-thienyl ketone was found. A methyl probe of the core revealed an opportunity for substituents at the R_2 position. Removal of a core nitrogen significantly reduced activity and the attachment of the amide side in the meta position was found to be optimal. This effort has helped to define the SAR for this interesting new series of antiproliferative agents which will be explored further in subsequent publications.

Acknowledgments

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References and notes

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11. *Cell proliferation assays*. p21+/+ and p21−/− cells were plated in 96-well tissue culture plates. The following day, dilutions of compounds were added and cells were cultured for 5 days. Cell survival was determined using sulforhodamine B, a protein binding dye. The concentration of the compound that inhibits cell proliferation by 50% (IC_{50}) was estimated from inhibition curves. The selectivity ratio is the ratio of the IC_{50} in the p21-proficient to the IC_{50} in the p21-deficient cells.
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15. Compounds were purified by HPLC and the purity was >90%. LC Conditions: HP 1100, 23 °C, 10 μL injected; Column: YMC-ODS-A 4.6 × 5.0 5 μm; Gradient A: 0.05% TFA/water, B: 0.05% TFA/acetonitrile; time 0 and 1 min: 98% A and 2% B; 7 min: 10% A and 90% B; 8 min: 10% A and 90% B; 8.9 min: 98% A and 2% B; Post time 1 min; Flow rate 2.5 mL/min; Detection: 215 and 254 nm, DAD. Semi-Prep HPLC: Gilson with Unipoint software; Column: Phenomenex C18 Luna 21.6 mm × 60 mm, 5 μM; solvent A: water (0.02% TFA buffer); Solvent B: acetonitrile (0.02% TFA buffer); Solvent gradient: time 0: 5% B; 2.5 min: 5% B; 12 min: 95% B; hold 95% B 3 min; flow rate: 22.5 mL/min; detection: 215 and 254 nm.
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